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(54) Title: VEGETARIAN PROTEIN FOODSTUFF

(57) Abstract: The present invention provides an edible foodstuff comprising protein based on fermentatively produced fungi whereby the fungal protein is enzymatically cross-linked.

# VEGETARIAN PROTEIN FOODSTUFF

## Field of the invention

The present invention relates to edible foodstuffs especially vegetarian protein foodstuffs comprising fungal derived protein which has been enzymatically cross linked and in particular to novel processes for the production of edible foodstuffs comprising fungal derived protein which has been enzymatically crossed linked. As a result, such foodstuffs have acceptable taste, texture and are devoid of any animal products and so are suitable for vegetarian and/or vegan consumption.

## Introduction

Animal meat always has been an important part of the human diet. However, due to a high number of food scares such as BSE, foot and mouth disease and dioxin contamination of animal products as well as due to an increasing concern about animal welfare, a rapidly growing number of people are turning to vegetarian or vegan diets, neither of which can include meat or meat derived products. Vegan diets are further characterized by the fact that they may not contain any product of animal origin, for example, eggs.

Apart from certain edible fungi (e.g. mushrooms) proteinaceous foods containing fungi are known. One example is the traditional Indonesian fermented food, tempeh. This is usually prepared by the fermentation of *Rhizopus* fungi with soy beans (and parts thereof) acting as a moist substrate. The beans (or other vegetable substrates) are inoculated with the fungus and fermentation allowed for 24 to 36 hours. The beans become bound by the fungal mycelium protein to give a firm product which can then be sliced before eating; no additional processing is usually performed before consumption.

The disadvantage of tempeh over meat is its lack of taste, flavour and fibrous and juicy texture which is associated with meat.

Thus a number of edible meat substitutes, meat replacements or novel protein foods have been proposed in recent years. Soy-based products, in particular

extruded soy, are marketed, but they do not have a particularly meat-like taste or texture (indeed both soy and gluten can have an "off" or astringent taste).

The art of making foodstuffs based on fungal mycelial protein (mycoprotein) has been developed on an industrial scale for the fungus *Fusarium graminearum* (EP-A-0123434). This fungus is used in meat substitutes sold in Europe under the brand name Quorn. During the preparation of these products egg albumin is added in order to obtain desired texture of various consumer products e.g. burgers, sausages, minced meat and the like. After the fermentation fungal biomass is separated from the medium by means of centrifugation. The resulting biomass is further processed by mixing with flavour and egg albumin. The latter addition is needed to bind the mycelia to form meat like fibres. Subsequently, the products is made by means of forming ,steaming, chilling and size reduction (slicing, dicing or shredding dependant upon the final type of consumer product).

Another example of an industrial process for the manufacture of mycoprotein and its subsequent use in food stuffs has been described for fungi of the order Mucorales in WO 00/15045 and WO 01/67886. The advantage of this process over that of *Fusarium graminearum* is the relatively lower RNA content in fungi of the order Mucorales. The RNA content of mycoprotein has to be below 2% to comply with food regulations.

However, for fungi of the order Mucorales one may also have to add egg albumin to bind the mycelia to obtain the right texture of meat like fibres. Egg albumin is derived from eggs which are produced by animals making this food unacceptable to vegans because of ethical objections. Eggs may also contain contaminants such as dioxin and human pathogenic bacteria such as Salmonella.

As far as we are aware it is unknown why the addition of egg albumin to fungal mycelia results in the formation of meat like structures.

Considering the above, there is a need for true vegetarian (or even vegan) protein sources with a better taste than soya or gluten based foodstuffs.

## Summary of the invention

The present invention provides an edible foodstuff comprising protein based on fermentatively produced fungi wherein the fungal protein has been enzymatically crossed linked. Such foodstuffs may or may not comprise a protein cross-linking enzyme.

The present invention also provides a process of production of an edible foodstuff, which comprises the addition of a protein cross-linking enzyme to an industrially produced fungal biomass containing protein.

## Detailed description of the invention

The present invention relates to the use of protein cross-linking enzymes, such as transglutaminase, to improve the texture of edible proteinaceous foodstuffs based on fungal cells. It is now possible to obtain a desirable texture without the addition of proteins of animal origin, for example the addition egg albumin to the fugal mycelium, during food preparation.

According to the present invention vegetarian protein foodstuffs based on fungal cells can be prepared which have an improved texture by the use of cross-linking enzymes derived from, produced by or present in a micro-organism.

To our surprise we have found that the addition of protein cross-linking enzymes to fungal biomass results in an acceptable texture of the protein upon forming, without the addition of egg albumin. This allows the manufacture true vegetarian protein foods based on fungal biomass.

Protein cross-linking enzymes are known. The cross-linking enzyme (e.g. transglutaminase) is preferably recombinantly produced, such as by heterologous expression of an encoding gene or cDNA in a suitable host organism, or, alternatively, by homologous (e.g. over expression) of a suitable endogenous gene. The enzyme may be produced by a (wild-type) strain of e.g. a *Streptoverticillium* species. Illustrative strains include *Streptoverticillium griseocarneum* IFO 12776, *Streptoverticillium cinnamoneum* sub sp. *Cinnamomeum* IFO 12852, *Streptoverticillium mobaraense* IFO 13819 and other species (cf. JP-A-64-27471). Alternatively, the enzyme may be produced (e.g. recombinantly by expression of a

heterologous gene) by a micro-organism such as a bacteria, yeast or fungus (e.g. filamentous fungi). Preferably the organism is of the genus *Streptomyces, Bacillus, Escherichia, Saccharomyces, Kluyvermyces, Hansenula, Pichia, Yarrowia, Candida, Aspergillus, Trichoderma, Penicillum, Mucor, Fusarium* or *Humicola*. Typical preferred (production) organims are *E. coli, Streptomyces* e.g. *Streptomyces lividans, Bacillus* e.g. *Bacillus licheniformis, Saccharomyces* e.g. *Saccharomyces cerevisae, Kluyvermyces lactis* and *Aspergillus* e.g. *Aspergillus niger*. The enzyme can be produced by fermentation (of the organism) and then additional processing to recover the enzyme.

Examples of protein cross-linking enzymes which can be used according to the invention comprise:

Transglutaminase (EC 2.3.3.13) ; protein-glutamine gamma-glutamyltransferase) is an enzyme capable of catalyzing acyl-transfer reactions introducing covalent cross-links between proteins as well as peptides and various amino acids. Large scale production of transglutaminase is possible by cloning a (microbial) gene encoding transglutaminase and expression in a suitable host.

There are a number of enzymes other than transglutaminase that are effective in cross-linking protein. Protein disulfide isomerase (EC 5.3.4.1), for example, catalyzes the reduction of disulphide groups in protein to reactive sulphydryl residues. Oxidation of these sulphydryl groups results in the formation of covalent cross-links between two polypeptide chains or between two residues in the same chain.

Sulphydryl oxidase (EC 1.8.3.2) or thiol oxidase is capable of catalyzing the oxidation of sulphydryl groups in proteins to disulfide bonds. This enzyme is found in both animals and microorganisms.

Polyphenol oxidase (EC 1.14.18.; previously classified as EC 1.10.3.1) is also known as catechol oxidase, tyrosine oxidase, tyrosinase, phenolase or phenol oxidase. This enzyme is widespread in nature and is found in many different types of animals such as e.g. mammals, insects, fish, moluscs, nematodes, plants and

microorganisms. The enzyme catalyzes the oxidation of phenols to ortho-diphenols which are subsequently oxidised to ortho-diquinone by the same enzyme. The ortho-diquinones react with sulphydryl groups or with amine groups within the protein to form protein cross-links.

Lysyl oxidase (EC 1.4.3.13) of lysyl-protein-6-oxidase is an important enzyme in the formation of protein cross-linking in collagen and elastin. Lysyl oxidase catalyzes the oxidative deamination of lysine to alpha-amino adipic-delta-semialdehyde or its hydroxide. These reactive molecules react with other amino acids to form cross links.

Peroxidase (EC 1.11.1.7.) is capable of catalyzing a large number of reactions in proteins in the absence or presence of hydrogen peroxide. Hydroxylation and peroxidation activities of peroxidase cause protein cross-linking. However cross-linking enzymes other than peroxidase(s) can be used depending on the circumstances.

Lipoxygenase (EC 1.13.11.12), also lipoxydase or linolate:oxygen oxidoreductase catalyses the oxidation of unsaturated fatty acids to fatty acid-peroxo radicals. These reactive molecules cause protein cross-linking.

Many kinds of oxidases produce hydrogen peroxide and cause protein cross-linking in an indirect way. Glucose oxidase (EC 1.1.3.4) for example, catalyses the oxidation of glucose to gluconolactone and hydrogen peroxide which in its turn oxidizes proteins.

Alcoholoxidases as e.g. methanoloxidase may result in protein cross-linking through the formation of aldehydes (formaldehyde in the case of methanol oxidase).

Alternatively, one may choose to add a polymeric or oligomeric carbohydrate with oxidizable groups, for example ferulic acid. Preferably plant or micriobal polymeric or oligomeric carbohydrates are used. Xylans present in, for example, wheat often contain such oxidizable groups. Addition of an oxidase to a medium

containing such carbohydrates will result in the cross-linking of proteins with this carbohydrate.

It is known from literature that certain proteases may carry out protein ligase and transferase reactions in the presence of high protein or peptide concentrations at relatively high pH. This so-called plastein reaction also results in protein cross-linking.

The fungal cells, fungal biomass or fungal protein is in general produced on industrial scale for example in a fermentor.

The fungi can be of the family *Choanephoraceae,* such as of the genus *Blakeslea* or *Gilbertella*, for example of the species *Blakeslea trispora* or *Gilbertella persicaria*. The other three families included within the order *Mucorales* are *Cunninghamellaceae, Mortiere llaceae* (such as fungi of the genus *Mortierella*, and in particular the species *Mortierella alpina*) and, especially, *Mucoraceae*. Suitable fungi are usually edible (and digestible) by humans or animals.

Preferred fungi are saprophytic (that is to say, simple fungi) rather than parasitic (which are more complex). The "simple" fungi are usually preferred because they are better adapted towards hyphal growth, whereas the parasitic organims concentrate on taking nutrients from their "host" organism.

The fungal cells are preferably of the genus *Rhizopus, Rhizomucor, Mucor* or *Mortierella*, all of which belong to the family *Mucoraceae*. Suitable fungal of the genus *Rhizopus, Mucor or Rhizomucor* include *Rhizopus stolonifer, Rhizopus miehei, Rhizopus pusillus, Rhizopus oligosporus* and in particular, *Rhizopus oryzae; Mucor hiemalis* and *Mucor rouxii*; and *Rhizomucor meihei*. Other preferred strains include those of the genus *Absidia* or *Phycomyces*, such as *Absidia pseudocylindrospora* or *Phyzomyces blakesleeanus*.

The addition of the protein cross-linking enzyme preferentially takes place during mixing of the fungal biomass and flavours but before forming and steaming. The steam-cooking after forming and addition of the cross-linking enzyme results in rapid inactivation of the enzyme activity. A complete inactivation of the enzyme is preferred in order to prevent further enzyme activity during consumption of the resulting product.

The present invention will now be described with reference to the following Examples.

## Comparative Example 1

### Generation of transglutaminase enzyme units

The method as described in WO 99/57993 was followed.

Transglutaminase from *Streptoverticillium sp.* was cloned and (over)expressed in *Streptomyces lividans* as described in US patent # 5,420,025. The *Streptomyces lividans* transformants expressing the *Streptoverticillium* transglutaminase gene were grown in a complex medium as described in this patent. The final fermentation broth had transglutaminase activities ranging from 1-10 U/ml

The microorganism was separated from the broth by means of centrifugation for 10 min. at 12,000 g at 4°C. The liquid containing the enzyme was concentrated by means of ultrafiltration (cut off was 1,000 Da) to a final enzyme concentration of 25 U/ml.

Enzyme analysis was then carried out as described in J.Biol.Chem. **241**: 5518 (1966) using benzyloxycarbonyl-L-glutamylglycine and hydroxylamine as substrates. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of one 1 micromole of hydroxamic acid per minute at 37°C.

## Comparative Example 2

*Rhizopus oryzae* (own isolate; other strains of this species are available from the Centraal Bureau voor Schimmelcultures, Delft, the Netherlands) was grown on labscale as described in WO 00/15045.

A spore suspension of the strain was prepared by growing the fungus for several days on a malt agar surface, rinsing the spores from the surface and storing them in a freezer. With this spore suspension an inoculum culture was started, using a soy bean meal based medium to promote hyphal growth (soy flour 15g/kg, yeast extract 5g/kg, $K_2HPO_4$ 1 g/kg and glucose. $H_2O$ 20g/kg). The medium was sterilized

for 45 min. at 120°C in Erlenmeyer flasks at pH 6. The flasks were incubated between 25 and 35°C for 2 to 4 days on an orbital shaker (with a 2.5 cm stroke at 250 rpm). As soon as full growth had been reached the culture was transferred to a lab fermentor containing a batch medium that was was prepared using the following components with indicated final concentrations:

Yeast extract (1g/kg), glucose (20g/kg), ammonium sulphate (6 g/kg), magnesium sulphate.$7H_2O$ (2 g/kg), calcium chloride (0.5 g/kg), potassium monophosphate (3 g/kg), zinc sulphate.$7H_2O$ (0.0144g/kg), iron sulphate (0.15 g/kg), manganese sulphate.$1H_2O$ (0.0288g/kg), copper sulphate.$5H_2O$ (0.0024g/kg), cobalt sulphate.$7H_2O$ (0.0038g/kg), thiamine.HCl (0.004 g/kg) and nicotinic acid (0.002 g/kg). All compounds were dissolved in deionized water and mixed, except the glucose and the phosphate which were sterilized separatetly. The pH was adjusted to 6.0 or 4.5 using NaOH and the medium was sterilized in the fermentor (10 L capacity) for 45 min. at 121°C, the first after acidification to pH 5 with phosphoric acid.

Next to the batch medium a carbohydrate feed was supplied which consisted of glucose at a concentration of ca. 500 g/kg. The preparation was as described for the glucose solution of the batch medium.

The fermentor was equipped with temperature, pH and foam controls. To adjust the pH, solution of ammonia and sulphuric acid were used. Dissolved oxygen concentration and the composition of the liberated gas were measured. The culture was aerated using ca. 1 volume of air per volume of broth per minute. Mixing was intensive using Rushton turbines and baffles. The glucose feed was applied at a rate between 1 and 5 g of glucose/kg borth/hour and started when the glucose concentration in the broth had decreased to a concentration below 5 g/kg. The temperature during fermentation was 30-35°C, the starting pH 4 and fermentation lasted for about 80 hours.

Samples were taken twice every 24 hours for off-line analysis of concentrations of unused substrate, biomass and by-products. Microscopic inspection was also performed. In all cases the biomass accumulated to concentrations from 30 to 40 g/kg within 80 hours of cultivation.

Directly after the end of fermentation, fungal cells were allowed to ripen. The temperature during this phase was maintained at 35°C for 3 hours with a constant pH of 4.5. Mixing continued for this period but the aeration was lowered to 0.1 volume of air per volume of broth per minute.

The biomass was separated from the broth by means of centrifugation (5 min. at 5000 rpm) and the biomass was then subjected to filtration on a lab filter press to remove excess water.

The resulting cake was washed with 10 cake volumes of water containing NaCl (0.9 g/L).

The cake was then frozen at -20°C for further treatment.

**Comparative Examples 3 and 5 + Example 4**

The filter cake from Comparative Example 2 was milled and crumbled by a high shear mixer for 5 minutes. The crumbled cake was subjected to the following three treatments:

a) addition of 4 g of egg albumin to 100 g of filter cake, a little water and spices (Comparative Example 3);
b) addition of transglutaminase ( in a range varying between 0.05 – 50 units) to 100 g of filter cake, a little water and spices (Example 4); and
c) addition of a little water and spices only (Comparative Example 5).

All three mixtures were homogenized in a kneader, kept for 30 min. at room temperature. Enzyme dosages vary between 10,000 and 10 U/kg of protein treated.

Patties were then made according to the following protocol: 100 g of the homogenized mixture was mixed with 4.6 g soy oil in a labscale food processor (Braun Combi type 700). The resulting dough was placed in moulds and heated to

85°C by steaming during 5 minutes. After chilling to 4-7°C the patties were frozen to -20°C.

A panel of 6 expert tasters judged the blind organoleptic properties of the three pattie samples after microwave heating. The panel judged patties made according to treatments a) and b) to be of acceptable taste and bite, whereas products made without the addition of egg albumin or a protein cross-linking enzyme, according to treatment c) where judged to be unacceptable due to a very poor bite sensation.

## Claims

1. An edible foodstuff comprising protein produced by fermentatively produced fungi wherein the fungal protein has been enzymatically cross-linked and/or the foodstuff additionally comprises a protein cross-linking enzyme.

2. An edible foodstuff comprising protein produced by fermentatively produced fungi which foodstuff is free of protein of animal origin.

3. An edible foodstuff according to claim 1 or 2 which is free of egg albumin.

4. An edible foodstuff according to any one of the preceding claims which is free of cross-linking enzyme activity.

5. An edible foodstuff according to any one of the preceding claims wherein the fermentatively produced fungal protein is derived from a *Rhizopus* species.

6. An edible foodstuff according to claim 1 in which the protein cross-linking enzyme is protein disulphide isomerase, protein disulphide reductase, sulphydryl oxidase, polyphenol oxidase, lysyl oxidase, alcohol oxidase, peroxidase, glucose oxidase or a transglutaminase or a combination thereof.

7. An edible foodstuff according to any one of the preceding claims wherein the protein cross-linking enzyme comprises a transglutaminase.

8. An edible foodstuff according to any one of the preceding claims wherein the enzyme is derived from, produced by or present in a microorganism or a plant.

9. An edible foodstuff according to any one of the preceding claims wherein the protein cross linking enzyme is of microbial origin and/or is a recombinant protein.

10. An edible foodstuff according to claim 8 or 9 wherein the enzyme is derived from, produced by or present in a microorganism which is a bacterium, yeast or fungus.

11. An edible foodstuff according to claim 10 in which the enzyme is derived from, produced by or present in a microorganism of the genus *Streptomyces, Bacillus, Escherichia, Saccharomyces, Kluyveromyces, Hansenula, Pichia, Yarrowia, Candida, Aspergillus, Trichoderma, Penicillium, Mucor, Fusarium* or *Humicola*.

12. An edible foodstuff according to claim 11 wherein the microorganism is *Streptomyces lividans*, *Escherichia coli*, *Bacillus lichenciformis*, *Kluyveromyces lactis* or *Aspergilus niger*.

13. An edible foodstuff according to claim 8 wherein the enzyme is contained in plant material which optionally has been obtained from a transgenic plant.

14. An edible foodstuff according to claim 13 wherein the enzyme is recombinant transglutaminase comprised in the seeds of a transgenic plant.

15. A process for producing an edible foodstuff according to anyone of the preceding claims which comprises the addition of a protein cross-linking enzyme to a fungal biomass containing protein.

16. A process according to claim 15 in which the protein cross-linking enzyme is transglutaminase.

17. A process according to claim 16 in which the transglutaminase is of microbial origin and/or is a recombinant protein.

18. A process according to any one of claims 15 to 17 wherein the cross-linking enzyme is inactivated following incubation with a fungal biomass containing protein.

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Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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